

THE ROLE OF THE CELL SURFACE PROTEINS OF *Burkholderia pseudomallei* IN THE SERODIAGNOSIS OF MELIOIDOSIS AND COMPARATIVE SEROLOGICAL PROTEOMIC ANALYSIS OF THE HUMORAL IMMUNE RESPONSE IN MELIOIDOSIS

ZAINOODIN SHEIK ABDUL KADER

UNIVERSITI SAINS MALAYSIA

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COMPARATIVE SEROLOGICAL PROTEOMIC ANALYSIS OF
THE HUMORAL IMMUNE RESPONSE IN MELIOIDOSIS**

by

ZAINOODIN SHEIK ABDUL KADER

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List of Presentations

The research findings arising from the experimental works carried out in this thesis were presented in part as a preliminary report at the following conferences.

Oral presentation

1. **Sheik Abdul Kader, Z.**, Aiyar, S. and Ismail, A. The Use of the Cell Surface Proteins of *Burkholderia pseudomallei* in Dot Immunoassay for the Serodiagnosis of Melioidosis. Paper presented at the “International Congress on Melioidosis: State of the Art Discoveries and Trends towards the 21st Century”. Siam City Hotel, Bangkok, Thailand. November 22-25, 1998.
2. **Sheik Abdul Kader, Z.**, Aiyar, S. and Ismail, A. Characterization and Comparative analysis of Antigenic Components of the Cell Surface Proteins of *Burkholderia pseudomallei*. Paper presented at the “World Melioidosis Congress”. Burswood International Resort Convention Centre, Pert, Australia. September 26th – 29th, 2001.
3. **Sheik Abdul Kader, Z.**, Aiyar, S. and Ismail, A. Serodiagnosis of Melioidosis: A Laboratory Perspective. Paper presented at the “National Diagnostic Conference and Workshop: Opportunities and Challenges in Medical Diagnostic Research”. Renaissance Kota Bharu Hotel, Kelantan, Malaysia. October 4th – 7th, 2004.

Poster presentation

Sheik Abdul Kader, Z. and Aiyar, S. The Efficacy of the Cell Surface Proteins of *Burkholderia pseudomallei* in the Serodiagnosis of Melioidosis. Poster presented at the “7th Western Pacific Congress of Chemotherapy and Infectious Diseases: Meeting the Challenges of the New Millennium”. Hong Kong Convention and Exhibition Centre, Hong Kong SAR, China. December 11th – 14th, 2001.

List of Abbreviations

2-D	Two-dimension electrophoresis
AP	Alkaline phosphatase
BCIP	C1-5-bromo-4-chloro-3-indolylphosphate p-toluidine
BSL3	Biosafety level 3 laboratory
CBB	Coomassie brilliant blue
CFA	Culture filtrate antigen
CSP	Cell surface proteins
DNA	Deoxyribonucleic acid
Dot EIA	Dot enzyme immunoassay
HIV	Human immunodeficiency virus
IEF	Iso-electric focusing
IFA	Indirect immunofluorescence antibody assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect hemagglutination assay
kDa	kilodalton
LPS	Lipopolysaccharide
MAPIA	Multi Antigen Print Immuno Assay
NBT	Nitroblue tetrazolium
NC	Nitrocellulose membrane
PAGE IEF	Polyacrylamide gel iso-electric focusing
PCR	Polymerase chain reaction
pI	Iso-electric point
rRNA	Ribosomal ribonucleic acid
SARS	Severe acute respiratory syndrome
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS-SM	Tris-buffered saline-skim milk
Tris	Tris(hydroxymethyl)aminomethane
WB	Western blot
www	World Wide Web

**PERANAN PROTEIN PERMUKAAN SEL *Burkholderia pseudomallei*
DALAM SERODIAGNOSIS PENYAKIT MELIOIDOSIS DAN
PERBANDINGAN TINDAKBALAS IMMUN HUMORAL
DALAM PENYAKIT MELIOIDOSIS MELALUI KAJIAN
SEROLOGIKAL PROTEOMIK**

Abstrak

Melioidosis adalah penyakit di kalangan manusia dan haiwan yang disebabkan oleh jangkitan bakteria, *Burkholderia pseudomallei*. Manifestasi klinikal penyakit ini amat rencam dan boleh berubah daripada keadaan yang tidak merbahaya kepada jangkitan teruk yang melibatkan anggota-anggota dalaman dan sekiranya tidak dirawat dengan segera boleh membawa maut. Kes akut perlukan rawatan dengan antibiotik yang sesuai untuk jangka waktu yang lama untuk mengelakkan jangkitan berkekalan (persistence) dan jangkitan berulang (relapse). Diagnosis klinikal selalunya tidak tepat disebabkan manifestasi klinikal yang tidak spesifik. Diagnosis makmal bergantung kepada kaedah pengkulturan dan pemencilan bakteria daripada spesimen klinikal. Kaedah pengkulturan kekal sebagai kaedah pilihan tetapi ia tidak sensitif dan memakan masa. Dalam dua dekad yang lalu, beberapa kajian untuk menghasilkan ujian serologi yang berkualiti untuk diagnosis segera melioidosis telah dilaporkan. Hasil daripada usaha ini kurang memuaskan.

Dalam kajian ini antigen protein daripada permukaan sel bakteria (CSP), *B. pseudomallei*, dengan dua kepekatan yang optimum telah digunakan untuk menghasilkan ujian enzim immuno titik ('dot enzyme immunoassay', 'dot EIA'). Protokol ujian 'dot EIA' yang piawai telah diwujudkan untuk diagnosis segera penyakit melioidosis akut dengan mengesan serentak kehadiran antibodi isotip IgM, IgG dan IgA dalam serum pesakit. Nilai sensitiviti yang diperolehi untuk mengesan setiap antibodi

isotip adalah seperti berikut: 47.7% untuk IgM, 89.2% untuk IgG dan 60% untuk IgA. Kualiti ujian ini bertambah baik apabila keputusan untuk ujian IgM, IgG dan IgA digabungkan di mana, sensitiviti meningkat kepada 95.5% dan spesifisiti meningkat kepada 92.5%. Dalam kajian prospektif, kes-kes melioidosis yang baru berjaya dikesan dan disahkan dengan ujian ‘dot EIA’ atas spesimen serum yang diambil daripada pesakit yang disyaki melioidosis. Kajian ini adalah yang pertama membuktikan potensi antigen CSP daripada *B. pseudomallei* untuk digunakan dalam ujian ‘dot EIA’ untuk diagnosis segera penyakit melioidosis dengan mengesan kehadiran antibodi isotip IgM, IgG dan IgA dalam serum pesakit.

Percirian antigen CSP dengan kaedah ‘Western blot’ (WB) konvensional telah mengesan tindakbalas serum melioidosis terhadap 25 jalur antigen dengan intensiti dan frekuensi yang berbeza. Keputusan perbandingan profil jalur-jalur antigen yang dihasilkan oleh 55 serum daripada pesakit melioidosis menunjukkan tiada satu profil jalur antigen yang mempunyai ciri-ciri diagnostik untuk melioidosis. Keputusan ini menggambarkan tindakbalas immun humoral pesakit melioidosis dalam jangkamasa jangkitan berbeza dengan ketara terhadap antigen-antigen yang terdapat dalam CSP. Profil protein antigenik yang dikesan dengan ujian WB konvensional telah dikategorikan dalam tiga kelompok. Kelompok 1 terdiri daripada jalur-jalur antigen yang berbentuk kembang dan tebal (‘broadly diffused bands’) dengan berat molekul antara 43 kDa hingga 36.8 kDa. Kelompok kedua merangkumi jalur-jalur antigen yang tersusun rapi (regularly spaced) dengan berat molekul 35.6 kDA hingga 25 kDa. Kelompok ketiga merangkumi jalur-jalur dengan berat molekul yang rendah iaitu antara 22 kDa hingga 13.2 kDa. Serum daripada subjek normal dan pesakit bukan melioidosis dari kawasan endemik menunjukkan tindakbalas silang yang agak ketara terhadap

antigen dalam kelompok 1 dan tindakbalas silang yang terbatas terhadap antigen dalam kelompok 2 dan 3. Ini menunjukkan individu yang sihat dan pesakit bukan melioidosis dari kawasan endemik mempunyai antibodi terhadap antigen CSP dan memiliki immuniti pada tahap yang signifikan terhadap melioidosis. Walaupun tindakbalas sera daripada kes melioidosis dan kes bukan melioidosis tidak dapat dibezakan dalam ujian WB terutamanya tindakbalas terhadap antigen dalam kelompok 1, perbezaan tindakbalas serum-serum ini pada peringkat epitop perlu ditentukan.

Dalam kajian serologikal proteomik, pemfokusan isoelektrik (IEF) dengan kaedah preparatif Rotofor IEF dan analitikal poliakrilamida gel IEF (PAGE IEF) telah digunakan untuk pencirian protein CSP berdasarkan kepada cas molekul. Pemisahan CSP dengan sistem preparatif Rotofor IEF telah menghasilkan 20 fraksyen rotofor. Satu keadah baru analisis immunoblot protein yang dipisahkan dengan kaedah PAGE IEF telah diwujudkan. Ujian immunoblot PAGE IEF yang telah dilakukan terhadap 20 fraksyen rotofor dengan serum melioidosis yang disatukan (pooled sera) menunjukkan kehadiran jalur antigen pada lokasi yang mempunyai nilai anggaran pI 4.0 hingga 6.5. Profil jalar-jalur antigen ini adalah yang berbentuk kembang, tebal dan beberapa jalur yang jelas (well defined bands). Analisis awal telah dijalankan ke atas fraksyen F16 dan F7 yang dipilih daripada fraksyen-frakyen rotofor yang reaktif untuk mengenalpasti kandungan protein dengan kaedah PAGE IEF. Protein yang telah dipurifikasi dalam matrik gel telah diekstrak dengan kuantiti yang banyak melalui sonikasi untuk kajian selanjutnya. Analisis awal dengan kaedah SDS-PAGE ke atas protein yang telah diekstrak menunjukkan kewujudan protein ‘isoform’ dan kejayaan dalam memperkayakan protein antigenik yang terdapat dalam jumlah yang sedikit serta terlindung dalam CSP. Kehadiran protein seperti ini tidak dapat dikesan dengan kaedah

WB konvensional. Protein ini juga telah menunjukkan tindakbalas yang kuat dan spesifik dengan serum melioidosis yang disatukan. Protein yang antigenik ini boleh digunakan bersama-sama dengan protein antigenik dalam CSP yang dikenalpasti melalui kaedah WB konvensional untuk menghasilkan ujian diagnostik multi-antigen yang lebih baik untuk penyakit melioidosis.

Kesimpulanya, kerja penyelidikan ini amat berguna untuk mewujudkan ujian serologi baru untuk diagnosis penyakit melioidosis. Selain itu, ia juga membina asas yang amat berguna untuk mengenal pasti mekanisme patologik dalam penyakit melioidosis, untuk mengenalpasti dan mendedahkan lebih banyak antigen untuk kegunaan diagnostik dan penghasilan vaksin untuk melioidosis.

THE ROLE OF THE CELL SURFACE PROTEINS OF *Burkholderia pseudomallei* IN THE SERODIAGNOSIS OF MELIOIDOSIS AND COMPARATIVE SEROLOGICAL PROTEOMIC ANALYSIS OF THE HUMORAL IMMUNE RESPONSE IN MELIOIDOSIS

Abstract

Melioidosis refers to an infection by the bacterium, *B. pseudomallei*, affecting man and animals. Clinical manifestations are protean, ranging from mild and self-limited diseases to progressive multi-organ infection with fatal consequences, if untreated. Treatment with prolonged course of antibiotics is required for the acute infection and the prevention of development of persistence of infection and relapse. The diagnosis based on clinical evidence is a challenging task, as the presenting symptoms mimic many other common infectious diseases. Direct isolation remains the mainstay in the laboratory diagnosis for melioidosis although it is less sensitive and time consuming. Over the last two decades, many experimental approaches towards the development of a reliable serological assay for rapid serodiagnosis of melioidosis have been reported. However, these attempts were often met with limited success.

In the present study, a standardized protocol of dot enzyme immunoassay (dot EIA test) with two optimized concentrations of the cell surface proteins (CSP) antigens from *B. pseudomallei* was established for serological diagnosis of melioidosis by detecting the IgM, IgG and IgA antibody isotypes as markers for current infection. The sensitivity of the dot EIA test for the detection of IgM, IgG and IgA antibody isotypes individually were 47.7%, 89.2% and 60% respectively. However, the overall combined detection of the IgM, IgG and IgA antibody isotypes improved the sensitivity and specificity of the dot EIA test to 95.5% and 92.5%, respectively. In addition, the dot EIA performed

satisfactorily in the prospective study for the diagnosis of melioidosis by detecting the IgM, IgG and IgA antibody isotypes in the sera tested from clinically suspected cases of melioidosis. The preliminary finding from this study demonstrated for the first time the potential use of the CSP of the *B. pseudomallei* as a source of antigens in the dot EIA test for early and accurate diagnosis of melioidosis by detecting the IgM, IgG and IgA antibody isotypes in the patient's serum.

Characterization of the SDS-PAGE separated CSP antigens by conventional Western blot (WB) analysis revealed 25 antigenic bands recognized by melioidosis sera with variable frequency and intensity. When the patterns of the antigenic bands were compared between the 55 sera from melioidosis patients, no single pattern was found to be diagnostically adequate. Great diversity in the humoral immune response against the CSP antigens was observed in each of the melioidosis patient during the time course of the infection. The heterogeneous antigenic protein profiles observed in the WB assay were categorized into three clusters of antigens. These included broadly diffused antigenic bands with the molecular weights ranging from 43 kDa to 36.8 kDa (cluster 1), regularly spaced antigenic bands with molecular weights ranging from 35.6 kDa to 25 kDa (cluster 2), and low molecular weights antigenic bands with molecular weights ranging from 22 kDa to 13.2 kDa (cluster 3). Some degree of comparable cross-reaction was observed between normal sera and non-melioidosis patients sera from endemic regions against the antigens in cluster 1. However, the endemic sera showed very limited cross-reactions with the antigens in the clusters 2 and 3. These data suggested that healthy individuals and non-melioidosis patients in endemic regions possessed antibodies specific to the CSP reflecting significant degree of background immunity to melioidosis. Although the reactivity of the non-melioidosis and melioidosis sera were

not distinguishable in the WB analysis especially against the antigens in the cluster 1, whether the non-melioidosis and melioidosis sera recognized similar and different epitopes present in the same antigens remains to be elucidated.

In serological proteomic study, the CSP was fractionated based on molecular charge in preparative Rotofor isoelectric focusing (IEF) and polyacrylamide gel IEF (PAGE IEF). Fractionation in the preparative Rotofor IEF produced 20 rotofor fractions. A new protocol to perform immunoblotting of the proteins separated by PAGE IEF gel was developed. The IEF immunoblot analysis of the 20 rotofor fractions with pooled melioidosis sera demonstrated presence of several strongly reacting bands located at the pI values approximately 4 to 6.5. Preliminary analysis was performed to determine the antigenic composition in the selected reactive rotofor fractions number F16 and F7 by PAGE IEF method. The fractionated proteins in the gel matrix were extracted in adequate quantity by sonication for subsequent analysis. Preliminary analysis by SDS-PAGE of the extracted proteins revealed the existence of the isoforms protein antigens and successful enrichment of the hidden low abundance antigenic proteins present in the CSP that otherwise may not be possible to detect by the conventional WB analysis. The purified proteins extracted from the selected fractions from IEF study showed strong and specific reaction with pooled melioidosis sera. These antigens can be used along with the antigens identified by the conventional WB assay of the CSP separated by SDS-PAGE to develop an improved multi-antigens based diagnostic assay for melioidosis.

In summary, this work would clearly be relevant for the development of newer serologic tests for diagnosis for melioidosis. Elucidation of the pathological mechanism in

melioidosis disease progression and recovery, exposition of more antigenic components for diagnostic application and development of vaccine for melioidosis would be other spin offs.

Chapter 1

General Introduction

1.1 Historical perspectives and present day importance of melioidosis

Melioidosis is an environmental related and potentially fatal bacterial infectious disease involving multi-organ systems of man and wide range of domestic and wild animals. It is caused by a soil saprophyte, *Burkholderia pseudomallei* (Chaowagul *et al.*, 1989, Leelarasamee & Bovornkitti, 1989, Sanford, 1995, Choy *et al.*, 2000, Josephson, 2001, Dance, 2002, O'Brien *et al.*, 2003). Documented historical evidence frequently quoted in the medical text and literature showed that a British surgeon, Captain A. Whitmore and his assistant, C.S. Krishnaswami were responsible for reporting the first case of melioidosis in Rangoon, the capital city of Myanmar (formerly known as Burma) in 1911 (Dance, 1991a, Sanford, 1995). These two medical professionals pioneered the historical discovery of melioidosis following a mysterious outbreak among the drug addicts. During routine postmortem work out, they discovered the presence of multiple abscesses mainly in the lungs, the liver, the spleen, and the kidneys in the dead bodies of the intravenous-drug abusers. The pathological features were noted to resemble glanders, an infectious disease caused by the bacterium, *Burkholderia mallei*. At that point of time, glanders was a well-recognized infectious disease of animals primarily in horses. They performed detailed clinical and bacteriological investigations following classic Koch postulate approach to resolve the unusual findings observed during the postmortem. They concluded the investigations with a report that described the pathology of the disease, the etiological agent and its clear distinction between glanders.

They also named the new agent as *Bacillus pseudomallei* and the disease was described as pseudoglanders (Howe *et al.*, 1971, Sanford, 1995). Shortly in 1913, Stanton and Fletcher from the United States Army Medical Research Unit at the Institute for Medical Research in Kuala Lumpur had reported severe distemper-like outbreak among the laboratory animals (Dance, 1991a). Subsequently in 1921, Stanton and Fletcher ascribed the infection to *B. pseudomallei*. They introduced the term melioidosis to collectively describe the entire clinical presentations arising from infection by *B. pseudomallei*. The term was derived from the Greek word in which ‘melis’ means ‘a distemper of asses’ and ‘eidos’ means resemblance (Dance, 1991a). Following these two historical records concerning melioidosis, only a few sporadic cases had been reported from various countries from the South-East Asia. The disease was not given priority compared to other well known infectious diseases at that point of time. Furthermore, in routine medical microbiology laboratories *B. pseudomallei* frequently isolated from clinical specimens was considered as contaminant.

During the military conflict in Indochina in the sixties, the occupying armies from United States of America and France apparently suffered significant loses due to melioidosis (Sanford & Moore, 1971, Dance, 1991a, Neel, 1991). Variability in clinical presentation, difficulties in concluding prompt laboratory diagnosis, and treatment complication with poor prognosis of melioidosis, were among the challenges faced by the medical professionals of the occupying armies in managing the soldiers during the wars (Dance, 1991a). This war marked the global resurgence of interest in melioidosis. The high incidence of melioidosis reported during the war associated with broad spectrum of clinical manifestations have evoked the scientific community and military authorities of the countries involved in the war to search for solutions to all aspects of

the melioidosis. Melioidosis also caused considerable concern to the military communities due to reports that it had potential to be used as biological warfare agent (Klietmann & Ruoff, 2001, Bruce, 2003). Currently extensive research is being carried out by scientist throughout the world for solution to the challenges and problems posed by melioidosis. As a result, International Congresses on Melioidosis have been held in Malaysia, Thailand and Australia in 1994, 1998 and 2002, respectively.

1.2 Global epidemiology of melioidosis

Melioidosis fall in the category of re-emerging infectious disease that poses a global threat to man and animals (Dance, 2000c). Sporadic cases and outbreaks are continuously being reported in the communities residing in the endemic regions. The global distributions of melioidosis by and large are restricted to tropical and subtropical regions especially in Southeast Asia and northern Australia as usually cited in several reviews and medical text. However, an alarming number of sporadic cases of melioidosis have increasingly been reported from non-endemic regions throughout the world. Several confirmed cases of melioidosis have been reported to occur in Taiwan, mainland of China and Hong Kong, Laos, Cambodia, Brunei, Vietnam, India, Bangladesh, Sri Lanka, Pakistan, Central and South America, North America, Africa, and the Middle East (Kang *et al.*, 1996, Dorman *et al.*, 1998, Parry *et al.*, 1999, Dance, 2000c, Yang, 2000, Hsueh *et al.*, 2001). Therefore the distribution of the disease is had not been restricted to the traditional endemic regions (Dance, 2000c, White, 2003). The boundaries of endemic regions are expanding out of the tropical and subtropical regions. Occurrences of melioidosis in non-endemic areas were largely due to increase mobility of people throughout the world and influx of immigrants from endemic to non-endemic

areas (Dance *et al.*, 1999). The disease was also recognised as potential health hazard to tourist and diplomat in the endemic countries (Thummakul *et al.*, 1999, Leelarasamee & Bovornkitti, 2001, Riesland *et al.*, 2001).

During the conclusion of the Vietnam war, serological survey conducted on war veterans showed the presence of low-titer hemagglutinating antibodies to melioidosis in as many as 225,000 US army veterans (Sanford & Moore, 1971, Bruce, 2003). Late onset of incidence of melioidosis associated with recrudescence had been reported from this group of infected veterans (Chodimella *et al.*, 1997). Small scale outbreaks had been reported in Brazil indicating wide spread occurrence of melioidosis throughout the world (ProMed-mail, 2003).

The incidence of the disease has increased over the last two decades in the traditional endemic regions that comprised of Singapore, Australia, Malaysia and Thailand. One reason for this could be the increased awareness of the disease among the medical and laboratory professionals. Other contributing factors would include the implementation of effective disease surveillance, improved diagnostic tools for effective laboratory isolation and identification, and mandatory disease notification.

The incidence in humans ranges widely between different endemic regions. There are several comprehensive reviews reported in the literature that described in detail the epidemiological investigations carried out in endemic and non-endemic regions throughout the world (Lim, 1997, Leelarasamee, 1998, Dance, 2000c, Finkelstein *et al.*, 2000, Leelarasamee, 2000, Faa & Holt, 2002, Cheng *et al.*, 2003). Incidence of

nosocomial infection associated with melioidosis also have been reported (Ashdown, 1979, White, 2003).

The epidemiological data also pointed out that the incidences of melioidosis were largely from tropical and subtropical regions with variable intensity of infection within and between the endemic countries. Variable intensity of infection within the endemic zone was attributed to segregated distribution of *B. pseudomallei* in the endemic area (Finkelstein *et al.*, 2000). Epidemiological surveys also identified the existence of two hyperendemic regions within the tropical zone, which comprised of the 'Top End' of the Northern Territory in Australia and Ubon Ratchatani and Khon Kaen provinces in north-east Thailand. Melioidosis was assigned as the most common community acquired infection in these two regions.

Incidences of melioidosis in Australia by and large are confined to the tropical regions which include Northern Territory, north Queensland, Torres Strait Islands and Western Australia (Currie *et al.*, 2001, Edmond *et al.*, 2001, Faa & Holt, 2002, Cheng *et al.*, 2003). Imported cases were reported in the temperate zone within this country (Wilson *et al.*, 1987). Epizootic incidences of animal melioidosis was found to be responsible for significant economic consequences in the livestock industry (Choy *et al.*, 2000). For the last two decades, the incidence of human infection was noted to be increasing in number.

In one study conducted in Torres Strait islands between 1995 and 2000 a total of 23 cases of melioidosis which represented a mean annual incidence rate of 42.7 per 100,000 population has reported (Faa & Holt, 2002). Similar study in Top End of the

Northern Territory from 1989 to 1998 revealed an average annual incidence rate of 16.5 per 100, 000 population (Currie *et al.*, 2000c). Recently an outbreak had been reported in Australia due to consumption of contaminated potable water supply (Inglis *et al.*, 1999, Currie *et al.*, 2001, Dance, 2002). Following this outbreak, a thorough study was carried out to determine the effect chlorine treatment on *B. pseudomallei*. The outcomes from the study showed that the bacteria had remarkable ability to survive in chlorinated potable water. Chlorine was found to have bacteriostatic effect on *B. pseudomallei* and the bacteria could be recovered from the treated water (Howard & Inglis, 2003b).

At present, it has been estimated that 2000–5000 patients per year present with melioidosis in Thailand (Beeching *et al.*, 2000, Leelarasamee, 2000, Dance, 2001). Several reports in the literature also pointed out that melioidosis might account for 29% of community-acquired septicemia and caused 40% of mortality among these patients in northeast Thailand (Dance *et al.*, 1989a). The northeastern Thailand comprising of Ubon Ratchatani and Khon Kaen were recognised as the main endemic regions for melioidosis. Parotid abscess was the most common feature of melioidosis among the children in Thailand (Dance *et al.*, 1989a). The seroepidemiological survey in northeastern Thailand revealed the presence of background immunity to melioidosis in 80% of children born by the age of 4 years (Leelarasamee, 1998).

In Singapore several exceptional features beside the common features found in Thailand and Australia have been reported. During the study period of 1987 and 1994, a total of 23 cases of melioidosis were reported in apparently healthy military personnel with four deaths (Lim *et al.*, 1997). In a separate study for the duration of 1989 to 1996 an alarming number of cases were also found in the community with disregard of their

professional and social status (Heng *et al.*, 1998). Every one in the community was found to have potential to succumb to melioidosis. During this period a total number of 372 cases were reported with 147 fatalities. The reported case fatality was alarming and considered much higher than total case fatality due to other common infectious diseases such as dengue fever, malaria, cholera and chicken pox. The exceptional features of melioidosis that were noted in Singapore included no correlation with well-recognized risk factors and rainfall. The disease occurred throughout the year and was not confined to any specific region. The overall incidence rates reported from epidemiological studies ranged from 1.7 to 2.4 per 100,000 population (Heng *et al.*, 1998, Goh, 2001).

1.3 Epidemiology of melioidosis in Malaysia

Historical document showed that Stanton and Fletcher pioneered the early work of human melioidosis in 1913 in Malaysia. Following that several seroepidemiological investigations documented in the medical literature that described in detail the high prevalence of melioidosis in many parts of the country (Sanford & Moore, 1971, Dance, 2000c). The reported epidemiological investigation pointed out the high prevalence of infection in the normal subjects. Moreover, melioidosis has been a well-recognised problem in the livestock industry. Several incidences of melioidosis in animals have been well documented by the Department of Veterinary Services, Ministry of Agriculture Malaysia (Mustaffa Babjee & Nor Aidah, 1994). However, the exact prevalence and incidence of melioidosis in human, and the true mortality rate have been difficult to establish for several reasons. There is no standardized statutory reporting of cases and melioidosis is not a in the list of notifiable disease. Owing to the high mortality rate, many fatal septicaemia cases could be due to melioidosis that remained

underdiagnosed. However, awareness regarding melioidosis in the community of infectious disease specialists, microbiologists and other healthcare workers leave much to be desired. In a thorough review on 50 cases of melioidosis admitted to a local Hospital, Puthucheary *et al.* (1992) clearly indicated the protean nature of the disease associated with high rate of mortality. She estimated that 15% to 20% of the Malaysian population has been infected with this bacterium (Oorjitham, 1998).

Recent reports in the literature has been shown an increase in the number of melioidosis cases in Malaysia (Yee *et al.*, 1988, Puthucheary *et al.*, 1992, Chang & Francis, 1994, Halder *et al.*, 1998, Puthucheary *et al.*, 2001).

1.4 General characteristics of *B. pseudomallei*

1.4.1 Classification

The genus *Burkholderia* has had a notable taxonomic history. Since its first description by A Whitmore in 1911, the status of the taxonomy and nomenclature of this bacterium has undergone considerable changes with multiple nomenclature schemes. The first name given by A. Whitmore was *Bacillus pseudomallei* (Howe *et al.*, 1971). Subsequently, several combinations of names were assigned to this bacterium, which included *Bacillus pseudomallei*, *Bacterium whitmori*, *Malleomyces pseudomallei*, *Loefflerella pseudomallei*, and *Pseudomonas pseudomallei*. Subsequently the organism was classified under the genus *Pseudomonas* that consists of five major groups based on rRNA-DNA homology. This organism was grouped under rRNA group II together with three related species (Sanford, 1995). In 1992, the taxonomist introduced a major

amendment to the nomenclature of this bacterium that led to moving it and seven other related organisms under new genus known as *Burkholderia* and the proposed type species for the genus was *Burkholderia cepacia*. Most of the members of the genus are found in the plant environment and are often responsible for diseases in plants. This clear classification and amendment was introduced based on the 16s rRNA sequence homologies, DNA-DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics (Yabuuchi *et al.*, 1994). The new genus takes its name after the name of an American plant microbiologist, Walter Burkholder, to honor his pioneer work on *Burkholderia cepacia* that was responsible for onion rot. However, the newly assigned genus was continuously reviewed and had undergone considerable changes. In a recent publication, a comprehensive study on genetic relatedness among the species in the genus was established (Coenye *et al.*, 2001). Following the restructuring exercise, the bacterium was officially named as *B. pseudomallei* and reassigned to the genus *Burkholderia* in the family *Burkholdericeae*, a member of subdivision of the *Proteobacteria*. Currently, the genus *Burkholderia* is composed of a heterogeneous collection of 22 species of which most are plant pathogens. This classification and differentiation of species was based on phylogenetic analysis of 16S rRNA genes. Phylogenetic features of two species in the genus *Burkholderia*, namely *B. mallei* and *B. thailandensis*, are very closely related to *B. pseudomallei* (Woo *et al.*, 2002, Godoy *et al.*, 2003). *B. thailandensis* was originally reported in Thailand and assigned as a less virulent arabinose positive biotype of *B. pseudomallei* (Sonthayanon *et al.*, 2002). After considerable debate and following an extensive genetic and phenotypic characterization the organism was renamed as *B. thailandensis* (Inglis *et al.*, 2003). The less virulent nature and the ability to utilize arabinose are the most distinctive features of this bacterium that differentiated it with *B. pseudomallei*.

1.4.2 Bacteriology of *B. pseudomallei*

B. pseudomallei is a Gram-negative, non-fermentative, non-sporing, capsulated, intracellular and motile bacterium. It can be seen singly, in pairs and occasionally in chains. The bacterium is non-fastidious, nutritionally versatile and able to grow well in any general basal medium. It is a free-living ubiquitous soil saprophyte found widely distributed in the natural environment in the endemic regions.

The most common natural niches associated with this bacterium include the surface water, streams, stagnant pond, ground water, damp soil, paddy field, play ground, rubber plantation, palm oil plantation, plants, including commonly consumed vegetables, newly cleared land and construction ground. It can be isolated from soil source at a depth ranging from 20 to 60 cm in endemic areas. During dry weather the organism can often persist for prolonged periods in clay layers of soil in the endemic areas without any form of nutrient (Dance, 1991b, Dance, 2000b, Inglis *et al.*, 2001). Findings from an in vitro experiment have shown the ability of this organism to penetrate and survive in free-living protozoa like acanthamoeba that are often present in collections of water. This observation indicates the possibility for a similar interaction that contributes to survival of *B. pseudomallei* in the environment and potential source for human infection (Inglis *et al.*, 2000).

The bacterium was also reported to have remarkable ability to survive for several years in nutrient limiting moist soil and water in tropical regions. It is also able to tolerate a range of deleterious environmental conditions such as effects of high acidic pH, freezing, drying, heat and currently practiced standard disinfection procedure for

potable water treatment. However, the bacteria are readily killed by a variety of disinfectants such as sodium hypochlorite at 500 ppm chlorine or 5% phenol with 10-15 minutes minimum exposure time.

Typical wrinkling colony morphology on the culture media can be seen after 48 hours of incubation. However, colony variation can be observed in culture, which include presence of small round and mucoid colonies or mixed population of small greyish and typical wrinkled colonies. The presence of atypical colony morphology that resembled the colony morphology of many other Gram-negative bacteria have been reported (Haussler *et al.*, 1999). Therefore, isolation of the bacteria from clinical specimens can be challenging due to colony variation. The organism can grow at 42°C and has the ability to survive at 4°C (Yabuuchi & Arakawa, 1993). Wide range of sugars and amino acid are utilised by the bacteria by oxidative or proteolytic reactions, respectively. Within the genus *Burkholderia*, the individual species can be differentiated on the basis of the physiological and biochemical reactions listed in table 1.1. These biochemical reaction properties are used in the routine laboratory identification of the bacteria. The important physiochemical properties used for identification of the culture isolate include ability to grow at 42°C, positive oxidase test, positive catalase test and production of nitrite and nitrogen gas from reduction of nitrate. Additional diagnostic tests for positive identification of the *B. pseudomallei* include positive Simmon's citrate reaction, non-fermentative reaction in TSI, resistance to polymyxin, colistin and aminoglycoside.

The whole genome sequencing task was carried out at The Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/B_pseudomallei/).

Table 1.1 Biochemical reactions for identification and differentiation of *B. pseudomallei* from the related species in the genus *Burkholderia*. Adapted from Zysk *et al.* (2000).

Compound	<i>B. pseudomallei</i>	<i>B. mallei</i>	<i>B. cepacia</i>	<i>B. vietnamensis</i>	<i>B. gladiolii</i>
Ribose	+	-	+	d	+
D-Xylose	-	+	d	+	+
L-Arabinose*	-	d	+	+	+
n-Valerat	+	-	+	+	+
2-Aminobenzoate	-	-	+	+	+
Malonate	-	d	+	d	+
L-Citrulline	-	-	+	d	d
Amylamine	+	-	+	d	-
Salicin	+	d	+	d	-
Pimelate	d	d	+	-	d
Glycolate	-	-	+	-	d
Citroconate	-	-	+	-	d
Tryptamine	-	-	+	-	-
Erythritol	+	-	-	-	-
D-Tartrate	-	-	-	-	+
Mesocionate	-	-	-	-	+
Itaconate	-	-	-	+	-
Glycine	-	+	-	-	-
L-Tartrate	-	-	d	-	+
Histamine	-	-	d	+	-
Sucrose	+	+	d	+	-
meso-Tartrate	-	-	d	d	+
Maltose	+	d	d	-	-

Abbreviation used in the table: +/-, more / less than 90% of the isolate yield a positive / negative reaction; d, between 10 to 90% of the isolates gave positive reactions; *, differentiates *B. pseudomallei* (-) and *B. thailandensis* (+).

In 2001, the sequencing process of the entire chromosomes of the *B. pseudomallei* was completed. The complete sequencing analysis was performed using the genomic material extracted from wild strain, K96243, a clinical isolate from Thailand. The genome size of the bacterium was estimated to be approximately 6 Mb comprising of two separate circular chromosomes with the size of 3.5 and 2.5 Mb, respectively (Songsivilai & Dharakul, 2000). Following successful completion of the whole genomes sequencing process of the bacterium, currently annotation work is being carried out at the centre (Sanger-Institute, 2001). Preliminary analysis of the complete genome sequences showed the presence of 1,041 open reading frames (ORFs). The genomes were also identified to be rich with guanine (G) and cytosine (C), which comprised of approximately 65% of the total genomes.

1.5 Mode of transmission

Melioidosis is a soil and water-borne disease and the well-documented mode of infection in human is through direct contact of injured skin or mucous membrane with contaminated soil or water (Chaowagul *et al.*, 1989, Dance, 2000a). Direct contact with infected livestock also has been proposed as source of infection especially to the abattoir workers. Furthermore, there are several reported cases of melioidosis in human that were acquired by other modes. These include documented cases of melioidosis infection acquired through respiratory route, ingestion, sexual and human-to-human contact (Achana *et al.*, 1985, Lee *et al.*, 1985, Kunakorn *et al.*, 1991, Abbink *et al.*, 2001, Currie *et al.*, 2001).

Those at risk of acquiring the infection are agricultural workers, plantation workers, farmers, construction workers, military personnel, gardeners, log cutters, abattoir workers and laboratory staff handling specimen. The epidemiological studies conducted in Australia and Thailand revealed that the seasonal peak of melioidosis were during or immediately after the rainy period (Inglis *et al.*, 2001, Currie & Jacups, 2003).

1.6 Clinical manifestations

Melioidosis is a progressive fatal infectious disease that imitates many other acute or chronic infectious diseases. The clinical presentations are extremely variable that can involve any organ system of the infected individual unlike a typical presentation commonly associated with many clinically important infectious diseases (Leelarasamee, 1998, Dharakul & Songsivilai, 1999). Therefore, many synonyms have been attributed

to this disease, which includes the great imitator, living time bomb, medical time bomb, Vietnam time bomb, Vietnam tuberculosis and silent killer. For these reasons, the definitive diagnosis based on clinical presentations is frequently difficult to establish. The outcome of infection is unpredictable and multiple host-related factors and properties of the infecting pathogen contribute to disease progression from mild flu-like infection to a rapid fatal septicaemia associated with widespread infection throughout the body. Despite the unpredictable clinical outcome of melioidosis, several reports quoted in the medical text and literature described an attempt to classify the clinical features of the disease (Sanford, 1995, Dance, 2001). Four main arbitrarily classified possible clinical consequences include asymptomatic subclinical infection, acute localized or widespread infection, subacute infection and chronic forms of infection (Ip *et al.*, 1995, Dance, 1996). However, as mentioned earlier, frequent overlap of all forms of clinical disease does occur. One form of disease may progress to another, and infections may present acutely with rapid progression to death or mild infection with chronic on going course associated with late sudden onset.

There are accumulating numbers of reports in the medical literature implicating several other anatomical sites with melioidosis which include skin, subcutaneous tissues, kidney, bones, joints, liver, spleen, brain, parotid glands, lymph nodes, pericardium, central nervous system, prostate gland, gastrointestinal system, musculoskeletal system and upper respiratory system (Woods *et al.*, 1992, Halder *et al.*, 1998, Currie *et al.*, 2000b, Karcher *et al.*, 2000, Edmond *et al.*, 2001, Tsang & Lai, 2001) Even though every vital organ can be infected but high tropisms to lungs have been indicated as a common feature of melioidosis.

The subclinical category of melioidosis represents the most common form of outcome encountered in a healthy individual that is often self-resolving. An infection in a healthy individual is usually associated with mild non-specific flu-like symptom and is often unrecognized. Majority of the infection occurring within the community in endemic regions fall in this category. Infected individuals in subclinical stage have circulating antibody. This complicates the interpretation of the serological tests. Several epidemiological studies have shown high rates of seroprevalence of melioidosis in endemic regions. A seroprevalence study conducted in Thailand showed the presence of antibody to melioidosis in 80% of the studied population comprising of children by the age of 4 years (Leelarasamee, 1998).

The acute form of melioidosis infection is characterised by three basic patterns, localised infection, pulmonary infection and disseminated acute septicaemia. Localized skin infections is characterized by the presence of abscesses, cellulitis and nodular form of skin infection that can spread to regional lymph nodes causing lymphadenitis and subsequent spread to organs such as the liver, spleen and lung. Other presenting features of the skin infection include persistent ulcers or sores that fail to heal and painful localized cutaneous nodular abscess at the site of infection.

Lung infection was the most common form of presentation of acute melioidosis that can give rise to a mild bronchitis to severe pneumonia. There are several unresolved non-specific clinical presentation often associated with this form of infection. These include unexplained fevers, weight loss, fatigue, cough, shortness of breath, headache, anorexia, and general muscle soreness. Chest pain is common, with a non-productive or productive cough. This may be confused with tuberculosis.

The more severe forms of melioidosis are often seen among people with underlying conditions that compromise the immune system. These conditions include diabetes, alcoholism, cancer, advanced age and chronic liver, kidney or lung disease. Drugs used to treat cancer and long-term steroid therapy can also impair the immune system. Disease in the infected individual with the underlying risk factors can progress from mild flu-like symptoms to fatal septicaemia associated with broad-spectrum of clinical diseases involving multi-organ systems. Non-specific symptoms can vary depending on the stage and severity of the infection. Any form of the infection can progress to fatal septicaemia with pyogenic abscesses involving multi-organ system and there is no definitive distinction of the disease stages (Howe *et al.*, 1971). The wide range of clinical presentations of acute melioidosis often resemble the symptoms of many other acute infections such as leptospirosis, typhoid fever, dengue fever, typhus, malaria, influenza, amebiasis, viral hepatitis, SARS (Bouadma *et al.*, 2003) and other infectious diseases commonly encountered in the tropical regions (Currie *et al.*, 2000a, White, 2003). High incidence of acute parotid abscess in apparently normal children was documented as a notable presenting feature in paediatric acute melioidosis cases in Thailand (Dance *et al.*, 1989a, White, 2003). The mortality rate of improperly treated acute septicaemia cases can be as high as 70–80%.

Subacute form of melioidosis is a progressive ongoing infection characterised by prolonged illness with or without fever associated with the presence of widespread multiple abscesses in almost every internal organs system with notable high tropism to respiratory system. The fourth category of clinical manifestation is chronic melioidosis which is the long standing suppurative abscess of internal organs system and the second most common presentation after the subclinical melioidosis. The clinical presentations

of chronic melioidosis are similar to the subacute infection but characterised by much longer incubation period than subacute infection in producing the clinical manifestations and may remain undetected for a number of years. The chronic form of melioidosis generally remains undiagnosed with minimum symptoms for many years until activated by a traumatic event or the presence of late-onset underlying diseases that impair the immune system.

1.7 Pathogenesis

The knowledge concerning pathogenesis and determinants of the disease severity for melioidosis are still scarce. The exact disease processes of melioidosis are yet to be defined clearly. Extensive research was engaged by many scientists throughout the world seeking new insights to the possible virulence determinant involved in the mechanism of the disease. Several animal models that resemble human melioidosis have been used to elucidate the pathogenesis of melioidosis (Hoppe *et al.*, 1999, Ulett *et al.*, 2001, Woods, 2002). Among the virulence factors implicated include the presence of flagella, capsular polysaccharide, extracellular proteases, endotoxin, exotoxin, haemolysin, lipase, acid phosphatase, siderophore, and presence of Type II O polysaccharide antigen that confer serum resistance to *B. pseudomallei* (Yang *et al.*, 1991, DeShazer *et al.*, 1998, Woods *et al.*, 1999, Brett & Woods, 2000, Lee & Liu, 2000, Reckseidler *et al.*, 2001, Chua *et al.*, 2003).

Similar studies were also reported that *B. pseudomallei* has the ability to penetrate, survive and multiply within not only in the professional phagocytes but also in virtually every nucleated cell of the body that enabled the infecting bacteria to cause wide spread

disseminated infection (Pruksachartvuthi *et al.*, 1990, Egan & Gordon, 1996, Jones *et al.*, 1996, Inglis *et al.*, 2000). The bacterium was found to adapt the type III secretion system to invade the host cell as well as to break out of the phagocytic vacuole to escape into the cytosol (Winstanley & Hart, 2000, Rainbow *et al.*, 2002, Stevens *et al.*, 2002). The type III secretion system is a well-characterised mechanism used by common pathogens such as *Salmonella*, *Yersinia* and *Shigella* to invade and damage the target cell by delivering the virulence factor straight into the host cell (Ehrbar *et al.*, 2003, Pujol & Bliska, 2003, Taniya *et al.*, 2003, Waterman & Holden, 2003). Once in the cytosol, the bacterium was found to induce polymerization of actin from the host cell cytoskeleton to form actin filaments at one end of the bacterium. This newly formed actin filament act like a driving force to push the bacterium against the host cell membrane which may protrude the cell surface and allow the bacteria to disseminate from cell to cell (Kespichayawattana *et al.*, 2000). This remarkable ability allows the bacteria to spread into the deeper tissues and lay dormant in the body for many years before recrudesce into an acute fatal infection. Additional virulence factors such as a capsular polysaccharide and low pH tolerance allows the bacteria to survive and lay dormant in the phagocytic cell for a prolonged period. In addition, chronic diseases such as diabetes, renal disease and malignancy contribute to decreased function of phagocytic cell which lead to vigorous intracellular growth that may trigger extensive immunopathological reactions that lead to severe clinical conditions in the susceptible host.

1.8 Differential diagnosis

The protean features of melioidosis made it difficult for its prompt diagnosis on clinical and laboratory ground. This has lead to melioidosis being named as the great imitator of many other infectious diseases. The confounding features include lack of distinctive clinical symptoms, the possible sudden onset of the clinical conditions, protean clinical manifestations, potential involvement of virtually any organ system with frequent unusual presentations and concurrent occurrence of melioidosis along with other diseases (Leelarasamee, 1998). Difficulties in laboratory diagnosis include low isolation rate, atypical colony morphology that often resemble colony morphology of other common bacteria, rapid overgrowth by normal flora and difficulties in accurate identification of culture isolate. Therefore, melioidosis must be considered in the differential diagnosis of a patient presenting with any febrile illness of unknown origin, multiple pustular skin or subcutaneous lesions, severe respiratory failure or when there is a chest x-ray pattern suggestive of tuberculosis but negative for acid-fast bacilli. A high index of suspicion and awareness are essential for early identification of potential cases of melioidosis particularly in patients presenting with the well-recognized risk factors or with a history of travel to or returning from well-known endemic regions.

1.9 Antimicrobial treatment and prognosis

Treatment of the disease poses considerable problems and is not a straightforward process like treatment for other common infectious diseases (Chaowagul, 2000). Furthermore, treatment and management of the disease are expensive, problematic, challenging and often annoying. Several studies have been reported regarding the

efficacy of antibiotic treatment and selection of a proper choice of antibiotic combination (Chetchotisakd *et al.*, 2001b, Apisarnthanarak & Little, 2002). It is a well-known fact that *B. pseudomallei* is innately resistant to penicillin, first and second generation of cephalosporins and aminoglycosides. In areas where melioidosis is endemic, the common empirical regimens that contain penicillin and gentamicin for the treatment of severe community-acquired pneumonia or septicaemia may not be appropriate.

In contrast to treatment for other common bacterial infections, treatment for the confirmed case of melioidosis comprised of two stages which include acute phase treatment and convalescent phase (eradication treatment). To some extent, the treatment protocol is comparable with treatment approach in tuberculosis. Initial stage involved treatment of acute infection for a minimum duration of two weeks but for more severe infection longer duration of treatment is required. Surgical procedure should be exercised whenever possible to drain abscesses. Patient recovered from acute infection must be put on an eradication treatment. To date, there is continuous debate over the best approach to convalescent and maintenance therapy to prevent recurrent infection (Simpson *et al.*, 1999, Simpson & White, 1999, Jenney *et al.*, 2001). No precise treatment guideline can be advocated for each category of clinical disease of melioidosis. Duration for eradication treatment can vary from 3 to 9 months depending on severity and clinical presentations during the acute infection.

Current recommendations for therapy of severe melioidosis include intravenous ceftazidime or imipenem for 10 days to 4 weeks, followed by eradication therapy with oral amoxicillin-clavulanate (Augmentin), or a combination of trimethoprim-

sulfamethoxazole and doxycycline for 10 to 18 weeks (White *et al.*, 1989, Chaowagul *et al.*, 1999, Chaowagul, 2000, White, 2003). However, the published reviews in the scientific literature recommended combination of four types of antibiotic regimens for effective eradication (Chaowagul *et al.*, 1999, Suputtamongkol *et al.*, 1999, Chetchotisakd *et al.*, 2001a, White, 2003). The four antibiotic regimens comprised of chloramphenicol, doxycycline and trimethoprim-sulfamethoxazole. However, for pregnant patients and children, chloramphenicol is contraindicated and the best option is amoxicillin-clavulanate (Augmentin).

Although the infection can be treated with readily available antibiotics but it is usually hard to achieve total elimination. Relapse, recrudescence, reinfection and reactivation are the common features of melioidosis (Chaowagul *et al.*, 1993). Despite complete optimal treatment, the prognosis is still poor. The overall mortality is 40% in severe melioidosis but varies depending on cases. It is greater than 50% for septicaemic disease and 20% for localized disease (Currie *et al.*, 2000c, Cheng *et al.*, 2003). High rate of relapse was attributed to persistence of intracellular infections that impede the treatment efficacy. Furthermore, ceftazidime, the antibiotic of choice for melioidosis is unable to kill the non-replicating cell of bacteria lying dormant in the phagocytic cell due to its poor cellular penetration.

A common problem arising from any treatment protocol prescribed for prolonged period is the emergence of resistant strain. Melioidosis belongs to this category of infectious diseases, therefore antibiotic resistance associated with treatment is another dilemma in patient management (Dance *et al.*, 1989b, Ho *et al.*, 2002, Tribuddharat *et al.*, 2003). The emergence of resistant strain of *B. pseudomallei* to ceftazidime during

treatment has been previously reported (Tribuddharat *et al.*, 2003). Hence, it has been noted in the literature that all confirmed cases of melioidosis require life long monitoring to detect emergence of resistant strain and clinical condition of the patient (Chaowagul, 1996, Jenney *et al.*, 2001).

1.10 Prevention

Currently there is no vaccine available for melioidosis (Brett & Woods, 2000). The disease is largely acquired through exposure to the environmental factors such as soil, water and vegetation (Heng *et al.*, 1998). Since the bacteria are widely distributed in the endemic areas prevention of the infection in such an environment have proven to be difficult. These have been indicated by several numbers of seroepidemiological studies that showed the presence of antibody to melioidosis in a large number of the population residing in the endemic areas or individuals travelling to the endemic areas (Chaowagul *et al.*, 1989, Currie *et al.*, 2000c, Finkelstein *et al.*, 2000, Yang, 2000, Riesland *et al.*, 2001, Dance, 2002). Several preventive measures have been proposed in the literature to contain the infection (Faa & Holt, 2002, ABC-News, 2004). These include a thorough body examination and cleaning after returning from outdoor activity in the endemic areas. Such a measure could reduce the risk of infection. Personal hygiene should never be neglected. Looking after personal hygiene practice is crucial and will greatly reduce the risk of getting infected especially in individuals associated with recognised risk factors.

Public health education also plays an important role in educating the public regarding the danger of the disease. The relevant health authority should emphasize on disease

prevention and implement concerted efforts in educating the public regarding the disease. The public should have the knowledge about the disease including awareness of the potential risk factors. They should also seek medical attention if facing any non-resolving prolonged fever associated with unknown causes. In health care settings, universal precautions in handling blood and body fluid should be implemented to prevent transmission of the diseases in the hospital or laboratory environments. Printed posters can be distributed to the public. Message also can be disseminated through public media, health educational brochures and newspaper.

1.11 Laboratory diagnosis

1.11.1 Routine culture isolation and direct microscopic examination of clinical specimens

Routine microbiological investigations in the laboratory diagnosis of melioidosis comprised of direct microscopic examination of Gram-stained smear of the clinical specimen and culture isolation of the organism. Gram-stained smear of various type of clinical specimens should be examined under direct microscopy. The presence of small Gram-negative rod-shaped organism with typical bipolar staining in direct microscopic examination should be considered as presumptive diagnostic indicator for melioidosis (Sanford, 1995, Walsh & Wuthiekanun, 1996).

Culture technique remains as a cornerstone for establishing the definitive diagnosis of melioidosis. The bacteria can be isolated from various clinical specimens, which include blood, urine, body fluid, sputum, throat, wound, stool or any purulent exudates. The